Molecular Ecology (2005) **14**, 2637–2643 doi: 10.1111/j.1365-294X.2005.02615.x

FAST-TRACK

Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*

A. BEHAR,*† B. YUVAL† and E. JURKEVITCH*
Department of Plant Pathology and Microbiology, †Department of Entomology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, 76100, Rehovot, Israel

Abstract

Nitrogen, although abundant in the atmosphere, is paradoxically a limited resource for multicellular organisms. In the Animalia, biological nitrogen fixation has solely been demonstrated in termites. We found that all individuals of field-collected Mediterranean fruit flies (*Ceratitis capitata*) harbour large diazotrophic enterobacterial populations that express dinitrogen reductase in the gut. Moreover, nitrogen fixation was demonstrated in isolated guts and in live flies and may significantly contribute to the fly's nitrogen intake. The presence of similar bacterial consortia in additional insect orders suggests that nitrogen fixation occurs in vast pools of terrestrial insects. On such a large scale, this phenomenon may have a considerable impact on the nitrogen cycle.

Keywords: *Ceratitis*, DGGE, diazotrophs, Insecta, *Klebsiella*, nitrogen fixation

Received 20 January 2005; revision received 22 March 2005; accepted 12 April 2005

Introduction

Nitrogen is a ubiquitous element, comprising 80% of Earth's atmosphere. Nevertheless, it is paradoxically a limited resource for many organisms (Dixon & Kahn 2004). Biologically available nitrogen originates from abiotic (about 3% from lightning and 30% from the fertilizer industry) and biotic inputs through biological nitrogen fixation mediated by diazotrophic bacteria (about 67% from both marine and terrestrial ecosystems) (Nardi *et al*. 2002). Diazotrophic bacteria are widely distributed in the prokaryotes, both within the Bacteria and the Archaea (Zinder & Dworkin 2000), reflecting the central role of this function in the nitrogen cycle. These bacteria are either free-living or associated with higher organisms. In the latter case, symbiotic associations have been demonstrated and thoroughly described in a few plant families (mainly legumes) (Vance 2002).

Many insects feed on nitrogen-poor diets (Slansky 1985; Waldbauer & Friedman 1991), and the origin of their nitrogen supply is also often poorly known. Therefore, it has been hypothesized that nitrogen fixation may be common within the Insecta and may contribute significantly to their nitrogen intake (Nardi *et al*. 2002). However, to date, the only insects known to harbour functional diazotrophic communities are the termites (Benemann 1973). Polygenetic analysis of both the 16S rRNA and dinitrogenase reductase (*nif* H) genes show that termite guts harbour a diversity of potentially nitrogen-fixing bacteria including *Citrobacter freundii*, *Enterobacter agglomerans*, and *Desulfovibrio* spp. (Ohkuma *et al*. 1999).

Fruit flies (Diptera: Tephritidae) are a highly successful, widespread group of insects causing enormous economic damage in agriculture. They are anautogenous, i.e. the acquisition of nitrogenous compounds by both male and female is essential for the realization of their reproductive potential (Drew & Yuval 2000). Although few data on the composition of the bacterial community found in the fruit fly digestive tract are available, members of enterobacterial species, mainly *Klebsiella* spp. and *Enterobacter* spp. were among the most often isolated bacteria (Drew & Lloyd 1987; Lauzon *et al*. 1998, 2000; Marchini *et al*. 2002). These species are known potential free-living diazotrophs, providing circumstantial evidence that nitrogen fixation may occur in these insects. Nevertheless, it has not been shown that these associations are constant and functional, i.e. that nitrogen fixation is actually taking place.

Accordingly, we tested the hypothesis that significant nitrogen fixation occurs in the gut of the Mediterranean

Correspondence: E. Jurkevitch, Fax: 972-8-9466794; E-mail: jurkevi@agri.huji.ac.il

© 2005 Blackwell Publishing Ltd

fruit fly *Ceratitis capitata*. Thus, we characterized the microflora of the Mediterranean fruit fly gut, determined the relative abundance of potential diazotrophs, established that these microorganisms express the dinitrogenase reductase gene, and demonstrated that nitrogen fixation takes place in live flies.

Materials and methods

Flies

Wild *Ceratitis capitata* flies were collected in traps containing water and figs during spring (April–June) and autumn (October–November) 2003 and spring 2004. They were dipped in a soap solution, quickly surface disinfected in 70% ethanol and washed in sterile phosphate saline buffer (PSB) prior to gut excision. The guts were sterilely extracted by dissection under a dissecting microscope and kept in 300 µL PSB. The guts were homogenized using a rotor-starter homogenizer and used to inoculate growth media or used for DNA and RNA extraction. Whole guts in PBS were used in the acetylene reduction assay.

Media and growth conditions

Nitrogen-fixing bacteria were enriched for using 10-fold dilutions of macerated individual guts in five replicates, by inoculating test tubes fully filled with a semisolid (0.05% agar), nitrogen-free medium (Burdman *et al*. 1998), and stoppered with a rubber plug. The tubes were incubated for 5 days at 30 °C. Colonies were isolated from pellicles on solid agar plates containing the same medium at 30 °C. For an estimation of total cultivable bacteria, dilutions of gut samples were used to inoculate tryptic soy broth in half-filled tubes, with shaking for 24 h at 30 °C. Most probable numbers were calculated using McGragy's tables (Postgate 1969).

Molecular methods and phylogenetic analysis

Total DNA was extracted from fly guts using a DNeasy Kit (QIAGEN) according to the manufacturer's instructions, from nitrogen-fixing pellicles, using a published procedure (Tsai & Olson 1991) and from isolated colonies by three successive cycles of freezing in liquid nitrogen and heating at 65 °C.

Polymerase chain reaction (PCR) was performed with eubacterial primers GC-clamp 968F-1408R, targeting the 16S rRNA gene (Heuer *et al*. 1997). Amplification was performed with 1 µm of each primer in 3 mm $MgCl_2$, 20 µm of each deoxyribonucleoside triphosphate, 1.25 units of *Taq* polymerase (Sigma) in a total volume of 50 µL of 1× reaction buffer (Promega). PCR was performed in a Mastercycler Gradient (Eppendorff) with a denaturation step of 2 min at 95 °C followed by 35 cycles at 95 °C for 20 s, 57 °C for 25 s, and 72 °C for 30 s, and a final elongation step of 72 °C for 1 min. The products were separated in a 1% (w/v) agarose gel in TAE buffer (40 mm Tris-acetate, 1 mm EDTA). Negative controls (no DNA added) were always performed in parallel. No products were obtained from these controls.

Denaturing gradient gel electrophoresis was performed using 35–55% and 40–60% urea-formamide gradients (Muyzer *et al*. 1993). Bands were extracted and directly sequenced at the Hebrew University Sequencing Center. Phylogenetic analysis was performed using the ARB package (Ludwig *et al*. 2004).

The *nif*H gene, encoding for the iron protein of the nitrogenase complex (Lilburn *et al*. 2001), was amplified from fly guts and from isolated colonies using one set of primers targeting *Enterobacteriaceae* species (expected product size *c*. 660 bp): NH1f (5′-ACACCATTATGGAGATGG-3′); NH1r (5′-GATGCCGAACTCCATCAG-3′) and one set specifically targeting *Klebsiella* spp. (expected product size *c*. 565 bp); NH2f (5′-GACTCCACCCGTCTGATT-3′); NH2r (5′-GTACTCGATAACCGTCATGC-3′). Amplification was performed as above with a denaturation step of 1 min at 94 °C followed by 25 cycles at 94 °C for 1 min, 53 °C for 45 s, and 72 °C for 2 min, and a final elongation step of 72 °C for 5 min. The products were separated in a 1% (w/v) agarose gel in TAE and purified using the High Pure PCR Purification Kit (Roche Molecular Biochemicals). A negative control (no DNA added) was run in parallel.

RNA was extracted using a Masterpure RNA purification kit (Epicentre) and reverse transcription was performed with the Improm-II reverse transcription system (Promega), both according to the manufacturer's instructions. cDNA synthesis and subsequent PCR amplifications of *nif*H were performed using both *nif*H primer sets as described above. A negative control (no reverse transcriptase added) and a positive control (*Klebsiella* DNA) were run in parallel.

PCR and reverse-transcriptase PCR products were extracted from the gels and cloned into a pGEM-T Easy Vector System I (Promega) according to the manufacturer's instructions. The vectors were transformed into competent *Escherichia coli* DH5α cells (Sambrook *et al*. 1989). Plasmids from selected colonies were purified with the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Clones were sequenced using the NH1f primer. Sequences were analysed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST).

Acetylene reduction assay

Acetylene reduction is used as a functional assay for nitrogen fixation, as the nitrogenase enzyme is capable of reducing the triple bond in acetylene, yielding ethylene, which is then detected by gas chromatography (Postgate 1978). Acetylene was produced by dissolving calcium

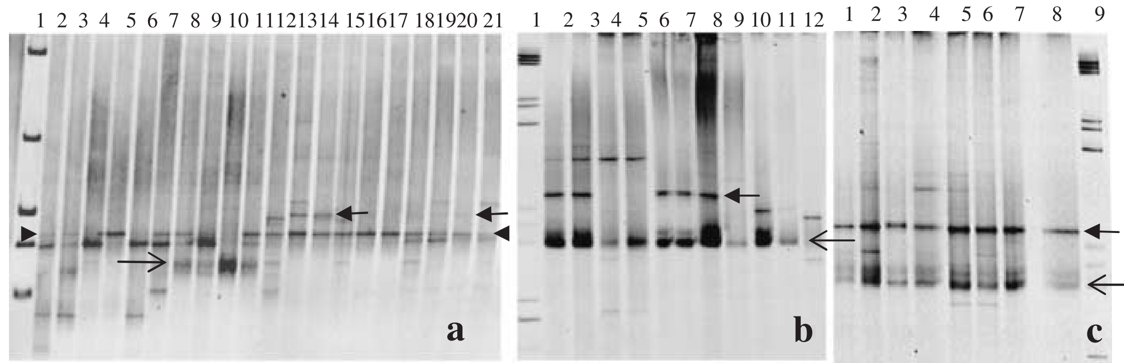

Fig. 1 Denaturing gradient gel electrophoresis of 16S rDNA PCR products obtained from (a) field-collected *Ceratitis capitata*. Pupae (lanes 2–6), larvae (lanes 7–11), adult male guts (lanes 12–16), and adult female guts (lanes 17–21). Marker (lane 1). (b) Pooled colonies obtained from adults' fly guts on tryptic soy agar: females (lanes 2–3, 6–7 and 10); males (lanes 4–5, 8–9, 11–12) in April (lanes 2–5), May (lanes 6–9) and July (lanes 10–12). Marker (lane 1). (c) Bacterial pellicles obtained from gut extracts and grown in a nitrogen-deficient, semisolid enrichment medium for nitrogen-fixing bacteria (lanes 1–8). Marker (lane 9). Products were run in a (a) 35–55%; (b and c) 40–60% urea-formamide gradient. Full black arrow: *Citrobacter freundii*. Open black arrow: *Klebsiella pneumoneae*. Black arrowhead: *Klebsiella* sp.

carbide in tap water. It was injected in closed vessels (15 mL) to a final concentration of 20% (v/v) by replacement of an identical volume of air. The vessels contained flies (experiment 1, $n = 12$; experiment 2, $n = 15$; experiment 3, $n = 17$), a CO_2 trap (2 N NaOH) and dessicated silica gel blue (Fluka, Germany) or a single colony isolated from nitrogen-fixing pellicles and grown on a nitrogen-deficient solid medium (in triplicate samples). Autoclaved flies were used as a negative control. Reduction of acetylene to ethylene was measured by flame ionization gas chromatography (Carlo Erba, Italy) after five hours of incubation at 30 °C. A molar ratio of 4 : 1 (ethylene: nitrogen) (Postgate 1978) was used to assess the amount of fixed nitrogen. To estimate the contribution of fixed nitrogen to the flies, we approximated a value of 16% nitrogen in protein (http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/x5557e/x5557e00.htm), and assumed that conditions conducive for nitrogen fixation by bacteria are, on average, 16 h per day.

Accession numbers (NCBI)

The 16S rDNA and *nif*H DNA and cDNA sequences determined in the present study are deposited at the GenBank under Accession nos AY847157 to AY847186, AY967762 and AY967763, respectively.

Results

Analysis of the gut's bacterial community structure

DNA was directly isolated from gut samples originating from field-collected males, females, larvae and pupae. Bacterial colonies were isolated from gut extracts on tryptic soy agar and DNA isolated from the pooled colonies. This provided a basis for a comparison between the cultivable and the total gut bacterial fractions. PCR products were obtained by the amplification of a fragment of the 16S rDNA and resolved by denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*. 1993), revealing that largely dominant populations occurred in both fractions (Fig. 1a–c). Populations detectable by PCR-DGGE usually constitute more than 1% of the total community, and major populations yield intense bands (Muyzer & Smalla 1998). Recurrent as well as sporadically appearing bands were extracted from several gels and sequenced. A phylogenetic analysis of these sequences showed that these bands originated from *Enterobacter* spp., *Citrobacter freundii*, *Klebsiella pneumoniae*, *Klebsiella* sp., *Pectobacterium carotovora*, and *Pectobacterium cypripedii* (Fig. 2). The most represented species belonged to the *Klebsiella*. *Klebsiella* sp. were present in all tested individuals ($n = 86$) at all developmental stages (larvae = 17; pupae = 17, adult males = 26; and adult females = 26) while *K. pneumoniae* was found in most of the insects examined (larvae = 4; pupae = 17; adult males = 19; and adult females = 25 females). *Citrobacter freundii* was only found in adult flies (adult males = 24; adult females = 22). Other species such as *Enterobacter* sp., *P. carotovora* and *P. cypripedii* were more sporadically detected by this direct analysis. Also, variations in banding patterns were observed, indicating that the population structure may shift during the season (Fig. 1b). Strikingly, all these bacterial species are known as potential nitrogen fixers (Ohkuma *et al*. 1999; Bell *et al*. 2004).

Frequency of diazotrophs' occurrence in the fly's gut

In order to assess the distribution and levels of potential diazotrophic bacteria in natural populations of Mediterranean

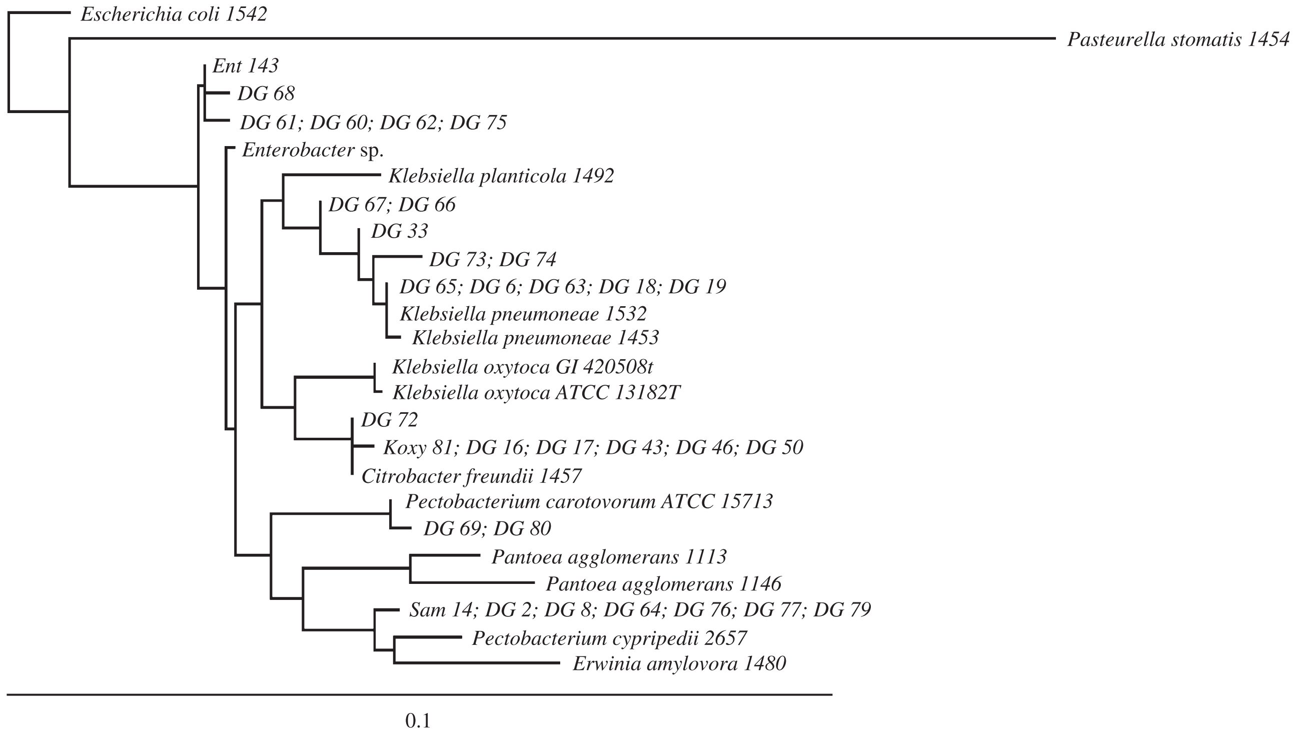

Fig. 2 Phylogenetic tree based on 16S rDNA sequence analysis of *Ceratitis capitata* gut bacteria. The tree is based on maximum-likelihood analysis, using a 10% conservation filter. Parsimony analysis essentially yielded the same topology. Scale bar indicates 10% estimated sequence divergence. Sequences extracted from denaturing gradient gel electrophoresis (DGGE) gels (*c.* 450 bp): DG69, DG80, DG66, DG73, DG33, DG65, DG63, DG6, DG72, DG68, DG75, DG62, DG2, DG8, DG77, DG64, DG76, DG79, DG43, DG46 and DG50 were obtained from the gut of field-collected flies; DG74 and DG61 were obtained from field-collected pupae; DG60 and DG67 were obtained from field-collected larvae and DG16, DG17, DG18, and DG19 from nitrogen-fixing pellicles. Sequences (*c.* 1350 bp) Sam14, Koxy81, and Ent143 were obtained from colonies isolated from pellicles. Homologous sequences from *Escherichia coli* and *Pasteurella stomatis* were used as outgroups.

fruit flies, extracts obtained from fly guts were serially diluted and inoculated in a semisolid enrichment medium for nitrogen-fixing bacteria. Extracts from all the individuals tested (males, $n = 27$; females, $n = 27$) yielded microaerophilic pellicles that developed below the agar surface, typical of diazotrophic growth. The level of diazotrophs was estimated at $1.10^8 \pm 3.7.10^7$ cells g^{-1} gut^{-1}. The size of the total cultivable fraction was similarly calculated to be $1.25.10^9 \pm 2.4.10^8$ cells g^{-1} gut^{-1} based on a parallel inoculation of the same dilution series in a general medium. The level of functional nitrogen fixers therefore was estimated at about 8% of the total bacterial population. This very high incidence corroborates the data obtained by DGGE analysis showing that these populations yielded intense bands in this molecular community analysis.

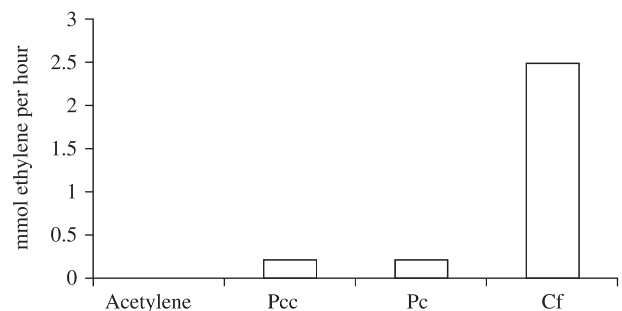

Fig. 3 Acetylene reduction assay conducted on pure cultures of *Citrobacter freundii* (Cf), *Pectobacterium cypripedii* (Pc), *Pectobacterium carotovorum* subsp. *carotovora* (Pcc) isolated from nitrogen-fixing pellicles. Negative control: 20% acetylene. Bars are not indicated as standard errors as they were too small to be drawn.

Colonies isolated from the pellicles and grown on a nitrogen-deficient medium were shown to significantly reduce acetylene to ethylene as compared to a negative control (one-way ANOVA: $F = 9.85e + 11$; $P < 0.001$) (Fig. 3), indicating they indeed are capable of nitrogen fixation. The almost full length sequences of the 16S rRNA gene amplified from these colonies were highly similar or identical to the sequences obtained by direct analysis of pellicle DNA (Fig. 1c), of DNA isolated from guts (Fig. 1a), and of pooled colonies isolated from the guts (Figs 1b and 2). We can therefore deduce that diazotrophic *Enterobacteriaceae* constitute a stable and major fraction of the microbial community of the fly's gut.

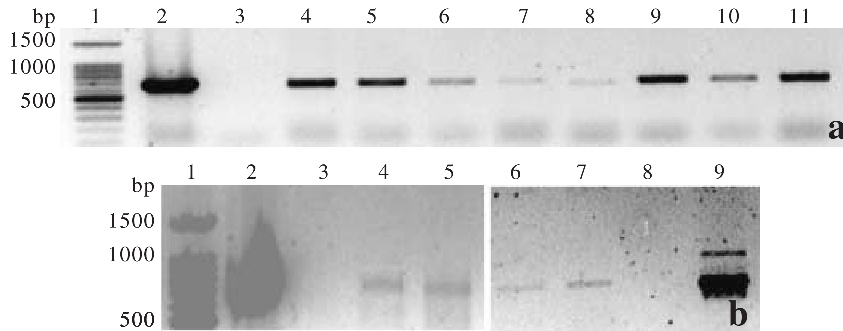

Fig. 4 Detection of (a) the *nif*H gene, using PCR and the *nif*H-specific primers NH1f-NH1r, in the guts of four adult males (lanes 4–7) and four adult females (lanes 8 to11). Pure culture of *Klebsiella* sp. (lane 2). No DNA added (lane 3). Molecular weight marker (lane 1), and (b) polymerase chain reaction (PCR) products of reverse-transcripted *nif*H mRNA from four replicates of pooled guts of field-collected *Ceratitis capitata* (lanes 4–7) using *nifH*-specific primers NH1f-NH1r. No reverse-transcriptase added (lanes 3 and 8). Pure culture of *Klebsiella* sp. (lanes 2 and 9). Molecular weight marker (lane 1).

nif*H analysis*

*nif*H was amplified by PCR from the gut of field-collected flies, from nitrogen-fixing pellicles, and from isolated colonies using one pair of primers specific for the *Klebsiella* genus (not shown) and one primer pair targeting *nif*H sequences of the *Enterobacteriaceae* (Fig. 4a). The presence of *nif*H strongly suggested that these bacterial populations are true nitrogen fixers. *In situ* expression of this gene was assessed using reverse-transcriptase PCR. A signal was obtained in pooled guts of adult field-collected flies ($n = 20$), thus providing supporting evidence that the nitrogenase complex was actively synthesized in the gut of the insect (Fig. 4b). Negative and positive controls (no RNA added and DNA added, respectively) yielded the expected results.

Cloning and sequencing of the products of the PCR and of the reverse-transcriptase PCR confirmed that they originated from *nif*H genes present and actively transcribed in the gut. A BLAST analysis showed that these sequences were 98% and 97% identical to the *K. pneumoniae nif*H gene, respectively.

Acetylene reduction in flies

Acetylene reduction, a functional assay for nitrogen fixation, was performed on groups of live field-collected adult flies (Fig. 5). Individuals were pooled ($n = 12–17$) and incubated in an acetylene-enriched atmosphere. While acetylene reduction into ethylene was detected in these pools of live flies, no such activity was present in pools of dead flies, or in an acetylene control without flies (18.0 ± 1.5; 6.7 ± 2.9 and 6.0 ± 2.9 nmol ethylene/h/fly, respectively) (one-way ANOVA: $F = 10.14$; $P = 0.002$). These data provided direct evidence that nitrogen fixation occurred *in vivo*. Moreover, acetylene reduction was detected in pooled isolated guts ($n = 5$) but not in the corresponding acetylene control

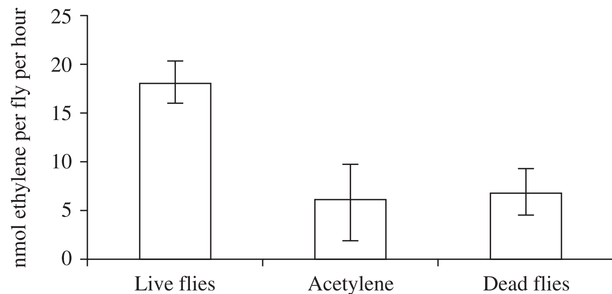

Fig. 5 *In vivo* measurement of nitrogenase activity. Acetylene reduction was performed on field-collected *Ceratitis capitata*. Negative controls: 20% acetylene without flies, and 20% acetylene with autoclave-killed flies. Bars represent standard errors.

(6.3 ± 0.32 and 3.07 ± 0.65 nmol ethylene/h/gut, respectively) (one-way ANOVA: $F = 19.92$ $P = 0.002$). It was estimated that the nitrogen equivalents of 6 μg of protein are fixed per fly per day.

Discussion

The data presented here demonstrate that nitrogen fixation occurs in *Ceratitis capitata* and results from the activity of stable and dominant populations of nitrogen-fixing *Enterobacteriaceae* located in the gut of this insect.

Our analysis of the Mediterranean fruit fly gut bacterial communities using both culture-dependent and culture-independent approaches revealed that *Enterobacteriaceae* constitute the most dominant populations in this environment as all the species identified in this analysis belonged to this family. Moreover, these species are known to be diazotrophs (Zinder & Dworkin 2000; Bergey *et al*. 2001).

Although most insects depend on nitrogen for reproduction, the sources of the nitrogenous compounds needed for their development and reproduction are not always

evident (Waldbauer 1968; Slansky 1993). In tephritid fruit flies bird faeces and bacterial cells may contribute to the nitrogen intake (Drew & Lloyd 1987), yet nitrogen is considered to be a factor limiting the reproductive success of both female and male Mediterranean fruit flies in nature (Yuval *et al*. 1998; Yuval & Hendrichs 2000).

Under nitrogen-deficient conditions, biological nitrogen fixation may provide the elemental demand required for growth, development and reproduction, as has been widely described in legumes (Vance 2002). Until now, termites were the only example in which nitrogen fixation has been demonstrated in the Animalia (Nardi *et al*. 2002). For such a process to occur and be ecologically important, the diazotrophic community must be widely distributed within the host population. Our results show that in *C. capitata*, at least one of the species of the free-living diazotrophs *Klebsiella* spp., *Citrobacter freundii*, *Enterobacter* spp. or *Pectobacterium* spp. (Zinder & Dworkin 2000; Bergey *et al*. 2001) is found at high levels in each fly at any developmental stage.

Diazotrophy in the host populations was supported by the growth of a microaerobic pellicle in a nitrogen-deficient medium and by the detection of the *nif*H gene in the gut. However, the presence of diazotrophs based on *nif*H detection must be treated with some caution (Burgmann *et al*. 2004). While a high incidence of nitrogen-fixing bacteria and the detection of *nif*H genes are prerequisites for functional and significant nitrogen fixation, these data do not prove the process actually occurs within the fly gut. This process is highly regulated at the transcriptional level by a sophisticated regulatory network that responds to multiple environmental cues (Dixon & Kahn 2004**.** Therefore, the expression of the gene was demonstrated by reverse-transcription PCR performed on RNA isolated from guts of flies captured in the field. Finally, demonstration of the occurrence of functional nitrogen fixation *in vivo* was provided by an acetylene reduction assay on live, field-collected flies.

We estimated that the nitrogen equivalents of 6 μg of protein are fixed per fly per day. In field-captured *C. capitata*, adult males contain between 35 and 40 μg of protein (Yuval *et al*. 1998), and in the laboratory females consumed about 15 μg protein per day (Galun *et al*. 1985). A similar amount of protein (*c*. 10 μg) was ingested by female Caribbean fruit flies (*Anastrepha suspensa*) in 5 h (Sharp & Chambers 1984). Also, most females of the Queensland fruit fly (*Bactrocera tryoni*), when fed *ad libidum* consumed 100 to 900 μg protein per day, depending on mating status (Meats & Leighton 2004). These data show that the amount fixed in *C. capitata* may provide a significant proportion of the fly's nitrogen requirements.

The bacterial species described in *C. capitata* were also retrieved from the guts of other insects such as the western flower thrip (*Frankliniella occidentali*s, Thysanoptera: Thripidae) (de Vries *et al*. 2001a, b), and other fruit flies such as *Rhagoletis pomonella* (Lauzon *et al*. 1998, 2000), *Rhagoletis completa* (Peloquin *et al*. 2000), *Anastrepha ludens* (Kuzina *et al*. 2001), and *Bactrocera tryoni* (Murphy *et al*. 1994). Moreover, in a long-term maintained laboratory strain of *B. tryoni*, nitrogen fixation could be shown after massive ingestion of *Klebsiella* and *Enterobacter* spp. cells (Murphy *et al*. 1994). All this suggests that nitrogen fixation may also occur in these insects.

Fruit flies are holometabolous, nonsocial insects found in plant canopies. Since the only other instance of proven biological nitrogen fixation in an animal is in termites, hemimetabolous, social soil insects, the cases cited above suggest that this phenomenon may be widespread within different orders of the Insecta. If, as we suspect, this relationship extends beyond the few species studied to date, it would provide massive amounts of fixed nitrogen ready to travel up the food chain by predation, down the food chain by decomposition, and to successive generations of the insect hosts by reproduction. A large-scale role for this process in insects, as well as its possible occurrence in aquatic habitats, such as coral reefs (Lesser *et al*. 2004), may change our views on the flow of nitrogen in nature.

Acknowledgements

We are pleased to thank Avi Levy, Saul Burdman and Susana Castro-Sowinski for their help with the manuscript. Our sincere gratitude goes to Carol Lauzon and Yaacov Okon for enriching discussions and to Batia Kaminski and Rahel Dror for technical assistance. This work was supported by the Binational Science Foundation (grant 2000-107-02) and by the Binational Agricultural Research and Development Fund (grant 3636-04).

References

Bell KS, Sebaihia M, Pritchard L *et al.* (2004) Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proceedings of the National Academy of Sciences, USA*, **101**, 11105–11110.

Benemann JR (1973) Nitrogen fixation in termites. *Science*, **181**, 164–165.

Bergey DH, Holt JG, Krieg NR (2001) *Bergey's Manual of Systematic Bacteriology* (ed. Garrity GM). Williams and Wilkins, Baltimore.

Burdman S, Jurkevitch E, Schwartsburd B, Hampel M, Okon Y (1998) Aggregation in *Azospirillum brasilense*: effects of chemical and physical factors and involvement of extracellular components. *Microbiology*, **144**, 1989–1999.

Burgmann H, Widmer F, Von Sigler W, Zeyer J (2004) New molecular screening tools for analysis of free living diazotrophs in soil. *Applied and Environmental Microbiology*, **70**, 240–247.

Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology*, **2**, 621–631.

Drew RAI, Lloyd AC (1987) Relationship of fruit-flies (Diptera, Tephritidae) and their bacteria to host plants. *Annals of the Entomological Society of America*, **80**, 629–636.

Drew RAI, Yuval B (2000) The evolution of fruit fly feeding behaviour. In: *Fruit Flies, Phylogeny and Evolution of Behavior* (eds Aluja M, Norrbom A), pp. 731–749. CRC Press, Boca Raton, Florida.

Galun R, Gothilf S, Blondheim S (1985) Comparison of aggregation and feeding responses by normal and irradiated fruit flies *Ceratitis capitata* and *Anastrepha suspensa* (Diptera: Tephritidae). *Environmental Entomology*, **14**, 726–732.

Heuer HK, Baker M, Smalla PK, Wellington EMH (1997) Analysis of actinomycetes communities by specific amplification of genes encoding 16S rRNA and gel-electrophoresis separation in denaturating gradients. *Applied and Environmental Microbiology*, **63**, 3233–3241.

Kuzina LV, Peloquin JJ, Vacek DC, Miler TA (2001) Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Current Microbiology*, **42**, 290–294.

Lauzon CR, Sjogren RE, Wright SE, Prokopy RJ (1998) Attraction of *Rhagoletis pomonella* (Diptera: Tephritidae) flies to odor of bacteria: apparent confinement to specialized members of Enterobacteriaceae. *Environmental Entomology*, **27**, 853–857.

Lauzon CR, Sjogren RE, Prokopy RJ (2000) Enzymatic capabilities of bacteria associated with apple maggot flies: a postulated role in attraction. *Journal of Chemical Ecology*, **26**, 953–967.

Lesser MP, Mazel CH, Gorbunov MY, Falkowski PG (2004) Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. *Science*, **305**, 997–1000.

Lilburn TC, Kim KS, Ostrom NE *et al.* (2001) Nitrogen fixation by symbiotic and free-living spirochetes. *Science*, **292**, 2495–2498.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Research*, **32**, 1363–1371.

Marchini D, Rosetto M, Dallai R, Marri L (2002) Bacteria associated with the oesophageal bulb of the medfly *Ceratitis capitata* (Diptera: Tephritidae). *Current Microbiology*, **44**, 120–124.

Meats A, Leighton SM (2004) Protein consumption by mated, unmated, sterile and fertile adults of the Queensland fruit fly, *Bactrocera tryoni* and its relation to egg production. *Physiological Entomology*, **29**, 176–182.

Murphy KM, Teakle DS, Macrae IC (1994) Kinetics of colonization of adult Queensland fruit-flies *Bactrocera tryoni* by dinitrogen-fixing alimentary-tract bacteria. *Applied and Environmental Microbiology*, **60**, 2508–2517.

Muyzer G, De Waal EC, Uitterlinder AG (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Applied and Environmental Microbiology*, **59**, 695–700.

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antoine van Leeuwenhoek International Journal of General and Molecular Microbiology*, **73**, 127–141.

Nardi JB, Mackie RI, Dawson JO (2002) Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? *Journal of Insect Physiology*, **48**, 751–763.

Ohkuma M, Noda S, Kudo T (1999) Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. *Applied and Environmental Microbiology*, **65**, 4926–4934.

Peloquin JJ, Kuzina L, Lauzon CR, Miller TA (2000) Transformation of internal extracellular bacteria isolated from *Rhagoletis completa* cresson gut with enhanced green fluorescent protein. *Current Microbiology*, **40**, 367–371.

Postgate JR (1969) Viable counts and viability. *Methods in Microbiology* (eds Norris JR, Ribbons DW), pp. 611–629. Academic Press, New York

Postgate J (1978) *Nitrogen Fixation*. Camelot Press Ltd, Southampton, UK.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sharp JL, Chambers DL (1984) Consumption of carbohydrates, proteins and amino acids by *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) in the laboratory. *Environmental Entomology*, **13**, 768–773.

Slansky JF (1985) Food utilization by insects: interpretation of observed differences between dry weight and energy efficiencies. *Entomologia Experimentalis et Applicata*, **39**, 47–60.

Slansky F Jr (1993) Nutritional ecology: the fundamental quest for nutrients. In: *Caterpillars Ecological and Evolutionary Constraints on Foraging* (eds Stamp NE, Casey TM), pp. 29–91. Chapman & Hall, New York and London.

Tsai YL, Olson BH (1991) Rapid method for direct extraction of DNA from soil and sediments. *Applied and Environmental Microbiology*, **57**, 1070–1074.

Vance CP (2002) Root-bacteria interactions: symbiotic N_2 fixation. In: *Plant Roots: The Hidden Half*, 3rd edn (eds Waisel Y, Eshel Y, Kafkafi U), pp. 839–868. Marcel Dekker, New York.

de Vries EJ, Breeuwer JAJ, Jacobs G, Mollema C (2001a) Growth and transmission of gut bacteria in the western flower thrips, *Frankliniella occidentalis*. *Journal of Invertebrate Pathology*, **77**, 129–137.

de Vries EJ, Breeuwer JAJ, Jacobs G, Mollema C (2001b) The association of western flower thrips, *Frankliniella occidentalis*, with a near *Erwinia* species gut bacteria: transient or permanent? *Journal of Invertebrate Pathology*, **77**, 120–128.

Waldbauer GP (1968) The consumption and utilization of food by insects. *Advances in Insect Physiology*, **5**, 229–288.

Waldbauer GP, Friedman S (1991) Self-selection of optimal diets by insects. *Annual Review of Entomology*, **36**, 43–63.

Yuval B, Hendrichs J (2000) Behavior of flies in the genus *Ceratitis* (Dacinae: Ceratitidini). In: *Fruit Flies, Phylogeny and Evolution of Behavior* (eds Aluja M, Norrbom A), pp. 429–456. CRC Press, Boca Raton, Florida.

Yuval B, Kaspi R, Shloush S, Warburg M (1998) Nutritional reserves regulate male participation in Mediterranean fruit fly leks. *Ecological Entomology*, **23**, 211–215.

Zinder DE, Dworkin M (2000) Morphological and physiological diversity. In: *The Prokaryotes*, 3rd edn (eds Dworkin M, Rosenberg E, Schleifer KH, Stackebrandt E). Springer Verlag, New York. [online.] http://www.prokaryotes.com.

Adi Behar is a PhD student. This work will constitute part of her thesis, under the supervision of B. Yuval and E. Jurkevitch. The Yuval laboratory studies behaviour and ecology of flies. The Jurkevitch group studies interactions between bacterial communities and eukaryotic hosts.